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Introduction

In breast cancers with *erb*B-2 overexpression abnormal cell proliferation is caused by the extremely active tyrosine kinase activity and resulting high level of signal transduction. An early and important intermediate in this signalling is the adaptor protein Grb2. This application proposed to design and test novel Grb2 inhibitors. This analysis will have important implications for translational cancer research by testing Grb2 inhibition as a target for therapy using compounds of defined biochemical activity.

Our approach was based on two discoveries. First, Dr. King and his associates have identified two peptides that are capable of binding to Grb2 and blocking its function. These peptides act to block the SH2 and N-terminal SH3 domain of Grb2. Second, publications report methods that facilitate introduction of peptides into cells. These studies show that membrane transit can be effected by fusion with a peptide sequence derived from either the signal sequence of protein that naturally crosses cell membranes or a sequence from the antennaepedia protein. Dr. King in his original application proposed to unite these approaches for the generation and testing of peptides capable of blocking growth factor signal transduction in human cancer cells.

The overall objective was to evaluate the phenotypic consequences of blocking Grb2 mediated signal transduction on the malignant phenotype of cancer cells that overexpress growth factor receptors such as p185*erb*B-2. We propose to measure the effectiveness of peptides in interrupting the targeted biochemical interaction in cell lysates and in whole cells and to correlate this intervention with changes in cell growth rate, cell migration, and anchorage independent growth. Since Grb2 signalling is part of diverse signal transduction pathways, we will also investigate whether Grb2 inhibitors effect cells that have no evidence of *erb*B-2 or other growth factor overexpression. In this way we test the hypothesis that Grb2 inhibitors have preferential activity for cancer cells with overexpression or activation of growth factor receptor tyrosine kinase activity. The validation of this hypothesis enhance the rationale for discovery, design, and pre-clinical testing of Grb2 inhibitors as cancer therapies. The support from USAMRMC for the peptide based studies has led to development of modified peptidomimetics and small molecule inhibitors and successful funding from the Komen Foundation for Breast Cancer Research.

The results from the peptide Grb-2 inhibitors study provided an important proof-of-concept for an approach that may generate specific, potent SH2 antagonists as clinical candidates in the near future. This type of SH2 antagonists might have a better therapeutic potential to be used either by themselves or in combination with other conventional chemotherapeutics in the treatment of breast cancer.

Specific Introduction for This Report.

Protein-protein interactions are involved in the regulation of virtually all aspects of physiological processes, for example, cellular signaling, and metabolic and transcriptional activation in cells (1, 2). Grb2 is an intracellular adaptor protein that consists of one SH2 domain and two SH3 domains, and mediates cellular signaling on growth factor (GF) receptor activation (3). Its Src homology 2 (SH2) domain binds to specific tyrosine phosphorylated motifs on activated GF receptors such as

EGFR and members of *erb*B family, and this leads to downstream activation of the Ras signal pathway which is highly relevant to a number of diseases including breast cancer (4, 5). Therefore, blocking the interaction between the phosphotyrosine-containing activated GF receptor and Grb2-SH2 domain provides a promising therapeutic target for development of new antitumor agents (6, 7). However, phosphotyrosine residue still serves as a critical recognition determinant for effective inhibitors binding to Grb2-SH2 (8-11). In the context of affording more bioavailability and specificity to binding (11-13), we developed a novel non-phosphorylated thioether cyclic peptide ligand (termed as G1TE, **1**) based on the original 1997 discovery of a phage library derived lead peptide (designated as G1) which bound to the Grb2-SH2 protein with 10-25 μM affinity, and was comprised of a 9 a.a. long sequence motif, $\mathbf{E^1\text{-}L\text{-}Y^3\text{-}E\text{-}N^5\text{-}V\text{-}G\text{-}M\text{-}Y^9}$, flanked by 2 terminal disulfide linked cysteines (14, 15). G1 was shown to lack binding propensity in the disulfide reduced open chain form, and it was found to be inactive under physiological conditions in inhibiting Grb2 / $p185^{erbB\text{-}2}$ association. On the other hand G1TE is redox stable, it exhibited equipotent binding affinity to G1, and was demonstrated to inhibit the association of the Grb2 protein with the growth factor receptor $p185^{erbB\text{-}2}$ in cell lysates derived from the breast cancer cell line MDA-MB-453 (14). Ala mutation studies on G1 indicated that essentially all amino acids, except Gly^7 in the peptide were necessary for retention of binding affinity (14), and Asn^5 and the unphosphorylated Tyr^3 were particularly important. G1 and G1TE define a new type of SH2 domain binding motif in a pTyr independent manner, but, at the same time our initial findings demonstrated that G1 requires a **YXN** sequence similar to that found in natural pTyr-containing ligands. We were intrigued to examine the functional importance and structural requirement of Tyr in particular in the consensus sequence of G1TE. We describe here a thorough study of the effect of incorporating various Tyr homologs and analogs, including tyrosine phosphate mimics on the binding affinity of the prototype peptide (Fig. 1). These studies provide an improved understanding of the molecular binding mechanism of this novel agent and suggest new strategies for designing potent non-phosphorylated inhibitors of Grb2-SH2 domain.

Body of Report

There were three technical objectives in the original proposal. The technical objective 1 was to generate cell permeable peptide inhibitors of Grb2. This has been done in collaboration with Dr. Peter Roller at Laboratory of Medicinal Chemistry of NCI. However, the initial fusion peptides made from the G1TE with a sequence from the antennaepedia protein failed due to the solubility and cell penetration problem. This may be peptide sequence specific problem since the other fusion peptides with the antennaepedia peptide worked well in other systems. We therefore made alternative efforts through a thorough study of the effect of incorporating various Tyr homologs and analogs, including tyrosine phosphate mimics on the binding affinity of the prototype peptide G1TE (Fig. 1). We have made twice more than the originally proposed peptide inhibitors. As shown in Tables 1 and 2, 14 peptides have been synthesized and their sequences were characterized by mass spectrometry.

The technical objective 2 was to determine the biochemical activity of cell permeable peptides. These include testing co-immunopreciptation inhibition method, testing effects of cell permeable peptides on co-immunopreciptiation of SOS1 and p185*erb*B-2 when peptides are added to cell

lysates, determining IC_{50} of inhibition in cell lysates, testing effects of cell permeable peptides on co-immunopreciptiation of SOS1 and p185*erb*B-2 when peptides are applied to intact cells and testing time course of activity of cell permeable peptide activity for Grb2 inhibition.. All of these objectives have been accomplished and are described in the following body of report.

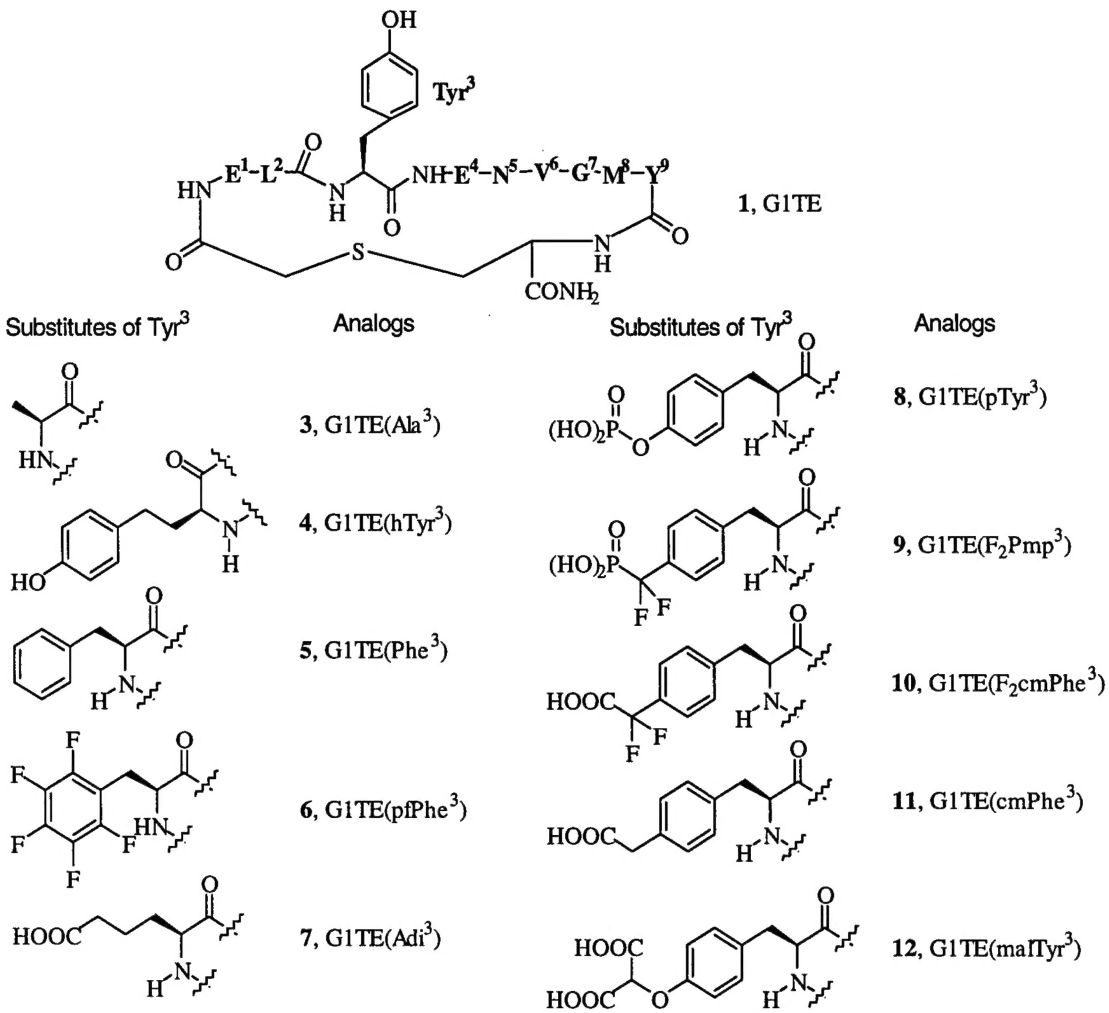

Figure 1. The structures of Tyr analogs and variants substituted at position 3 in G1TE.

The technical objective 3 was to determine effects of cell permeable peptides on cancer phenotype. Part of this objective has been accomplished and is also described in the body of this report.

Design and Synthesis of Modified Grb2 Inhibitors. The synthesis of the cyclic thioether peptides **1-14** was carried out in a convenient manner, similar to that previously reported (16, 17). Briefly, the linear peptide was synthesized on solid phase on a PAL amide resin with an ABI 433A peptide synthesizer utilizing FastMoc chemistry. After removing the N^{α}-Fmoc group with 20% piperidine/DMF, the resin-bound protected peptide was N-terminally chloroacetylated by $(ClCH_2CO)_2O$, which was prepared by mixing 0.5 M $ClCH_2COOH$/DCM and 0.5 M DCC/DCM for 1 h at RT, and filtering off the precipitated DCU. Chloroacetylation was carried out for a

duration of 6 hrs at RT, or until the Ninhydrin Test proved to be negative. The open chain peptide was cleaved from the resin by using TFA containing 2.5% each (v/v) of triethylsilane and deionized water (2 h). For isolation of the product, two-thirds of the cleavage reagent mixture was evaporated under N_2 and the mixture triturated in ice-cold ether. For cyclization the precipitated crude peptide was dissolved in 50 mL of water and added dropwise into 100 mL of aqueous solution, which was adjusted to pH 8~9 with triethylamine, repeatedly. Under the basic conditions the N-chloroacetylated linear peptide cyclized spontaneously by intramolecular nucleophilic displacement of the chloro group by cysteine thiol. The final product was purified by RP-HPLC, and the identity was assessed by amino acid and mass spectral analyses (see Table 1).

Table 1. The physicochemical data of peptides **1-14**

Peptide	RP-HPLC[a]	FAB-MS[b]	Amino Acid Analysis
1	R_t = 12.8 min (gradient 20-80% B over 30 min, I)	$(M+H)^+$ 1258.7 (calc. 1259.5)	Asp 1.418(1), Val 1.348(1), Leu 1.345(1), Glu 2.768(2), Gly 1.384(1), Tyr 2.508(2), Met 0.997(1)
2	R_t = 13.9 min (gradient 20-60% B over 30 min, I)	$(M+H)^+$ 1096.8 (calc. 1097.3)	Asp 1.13(1), Val 0.98(1), Leu 1.17(1), Glu 1.78(2), Gly 1.01(1), Tyr 0.65(1), Met 1.01(1)
3	Rt = 12.7 min (gradient 20 ~ 80% B over 30 min, I)	$(M+H)^+$ 1166.0 (calc. 1167.3)	Asp 0.61(1), Val 1.00(1), Leu 1.23(1), Glu 2.05(2), Gly 1.20(1), Ala 1.07(1), Tyr 0.63(1), Met 0.88(1)
4	R_t = 14.1 min (gradient 20-70% B over 27 min, I)	$(M+H)^+$ 1273.8 (calc. 1273.5)	Asp 0.55(1), Val 1.05(1), Leu 1.21(1), Glu 1.93(2), Gly 1.34(1), Tyr 0.73(1), Met 0.85(1)
5	R_t = 15.9 min (gradient 20-80% B over 30 min, I)	$(M+H)^+$ 1244.2 (calc. 1244.5)	Asp + S-CM-Cys 1.14(1 each), Val 1.22(1), Leu 1.34(1), Glu 1.94(2), Gly 1.25(1), Tyr 0.75(1), Met 0.72(1), Phe 1.19(1)
6	R_t = 16.3 min (gradient 20-70% B over 27 min, I)	$(M+H)^+$ 1334.6 (calc. 1334.4).	Asp 1.10(1), Val 0.97(1), Leu 1.15(1), Glu 1.80(2), Gly 1.05(1), Tyr 0.66(1), Met 0.97(1)
7	R_t = 12.8 min (gradient 20-70% B over 27 min, I)	$(M+H)^+$ 1239.6 (calc. 1239.5).	Asp + S-CM-Cys 1.81(1 each), Val 1.09(1), Leu 1.19(1), Glu 1.93(2), Gly 1.09(1), Adi 0.88(1), Tyr 0.84(1), Met 1.01(1)
8	R_t = 13.3 min (gradient 10-70% B over 25 min, I)	$(M+H)^+$ 1339.8 ($calc._{ave}$ 1338.5)	Asp + S-CM-Cys 1.91(1 each), Val 1.13(1), Leu 1.17(1), Glu 1.65(2), Gly 1.20(1), Tyr 1.65(2)*, Met 0.96(1)
9	R_t = 11.0 min (gradient 20-60% B over 30 min, I)	$(M+H)^+$ 1373.6 (calc. 1373.5)	Asp + S-CM-Cys 1.31(1 each), Pro 1.42(1)*, Val 1.21(1), Leu 1.15(1), Glu 1.93(2), Gly 1.19(1), Tyr 0.83(1), Met 0.63(1)
10	R_t = 13.9 min (gradient 20-70% B over 27 min, I)	$(M+H)^+$ 1337.3 (calc. 1337.5)	Asp + S-CM-Cys 1.68(1 each), His 1.77(2)*, Val 1.20(1), Leu 1.32(1), Glu 2.08(2), Gly 1.21(1), Tyr 0.73(1), Met 0.77(1)
11	R_t = 16.3 min (gradient 10-70% B over 27 min, II)	$(M+H)^+$ 1361.8 ($calc._{ave}$ 1362.5)	Asp + S-CM-Cys 1.48(1 each), Val 1.16(1), Leu 1.27(1), Glu 1.97(2), Gly 1.14(1), Arg 1.38(1)*, Tyr 0.69(1), Met 0.77(1)

	over 27 min, II)		
12	R_t = 13.7 min (gradient 20-70% B over 27 min, I)	$(M+H)^+$ 1302.2 (calc.$_{ave}$ 1302.4)	Asp + S-CM-Cys 1.58(1 each), Val 1.13(1), Leu 1.21(1), Glu 1.86(2), Gly 1.17(1), Tyr 0.89(1), Met 0.97(1)
13	R_t = 18.7 min (gradient 10-70% B over 27 min, II)	$(M+H)^+$ 1243.1 (calc. 1243.5)	Asp + S-CM-Cys 1.17(1 each), Val 0.95(1), Leu 1.09(1), Glu 0.94(1), Gly 1.12(1), Ala 0.96(1), Tyr 0.70(1), Met 0.68(1)
14	R_t = 18.5 min (gradient 10-70% B over 27 min, II)	$(M+H)^+$ 1315.6 (calc.$_{ave}$ 1315.5)	Asp + S-CM-Cys 1.24(1 each), Adi 0.91(1), Val 1.06(1), Leu 1.13(1), Glu 0.96(1), Gly 1.05(1), Tyr 0.75(1), Met 0.65(1)

[a] HPLC conditions I: Vydac C18 column (10x250 mm); solvent gradient, A, 0.05% TFA in water; B, 0.05% TFA in 90% acetonitrile in water with gradient indicated; flow rate, 2.5 mL/min; UV detector, 225 nm. HPLC conditions II: Vydac C4 column (20x250 mm); solvent gradient, A, 0.05% TFA in water; B, 0.05% TFA in 90% acetonitrile in water with gradient indicated; flow rate, 10.0 mL/min; UV detector, 225 nm.
[b] FAB-MS (unit resolution, glycerol matrix) was performed on a VG Analytical 7070E-HF mass spectrometer.
[c] Amino acid analysis (6N HCl, 100 °C, 24 h) was carried out at the Protein and Carbohydrate Structure Facility (University of Michigan, Ann Arbor, Michigan, USA).
* For peptides containing nonstandard amino acids, additional unassigned HPLC peak was observed.

The PAL amide resin and Fmoc derivatives of standard amino acids were obtained from Perkin-Elmer/Applied Biosystems Division (Foster City, CA, USA). L-homoTyr (hTyr), L-α-aminoadipic acid (Adi) and O-malonyl-L-Tyr (malTyr) were purchased from BACHEM (Torrance, CA, USA) in Fmoc protected form. N^{α}-Fmoc-L-Tyr(PO(OH,Obzl))-OH from NOVAbiochem (La Jolla, CA, USA) was used for the synthesis of phosphotyrosine-containing analogs. Fmoc-L-2,3,4,5,6-Pentafluoro-Phe-OH (pfPhe) was purchased from Synthetech Inc. (Albany, OR, USA). Phosphotyrosyl mimetics 4-carboxymethyl-L-phenylalanine (cmPhe) and 4-carboxydifluoromethyl-L-phenylalanine (F_2cmPhe) were prepared with O-tBu sidechain protection and N-terminally Fmoc protected (18). N^{α}-Fmoc-4-phosphonodifluoromethyl-L-phenylalanine (F_2Pmp) was synthesized with sidechains unprotected (19).

Binding affinity measurement of peptides to Grb2-SH2 domain using Surface Plasmon Resonance (SPR). The competitive binding affinity of ligands for the Grb2-SH2 protein was assessed by using Biacore SPR methods. On a BIAcore 2000 instrument (Pharmacia Biosensor, Uppsala, Sweden). IC_{50} values were determined by mixing various concentrations of inhibitors with the recombinant GST-Grb2-SH2 domain protein and measuring the amount of binding at equilibrium to an immobilized SHC phosphopeptide($pTyr^{317}$), i.e. biotinyl-DDPS-pY-VNVQ, in a manner described previously (14). The biotinylated phosphopeptide was attached to a streptavidin coated SA5 Biosensor chip, and the binding assays were conducted in pH 7.4 PBS buffer containing 0.01% P-20 surfactant (Pharmacia Biosensor).

Molecular Modeling. The Insight II 97/Discover 3.0 modeling package from Molecular Simulations Inc., San Diego, CA with the cff91-force field was used. The X-ray structure of the KPFpYVNV peptide ligand bound to the Grb2-SH2 was used as a starting geometry (20). The positions of the backbone atoms, and of the sidechain atoms of those residues in the turn region of the reference peptide sequence, -F-pY-V-N-V-, that are identical in G1TE(Adi^1, $cmPhe^3$) were

used as the initial atom positions of the sub-sequence, -L-cmPhe-E-N-V- in G1TE(Adi^1, cmPhe^3), since a turn has been predicted for the related subsequence in G1TE also (21). Then, the remaining residues of G1TE(Adi^1, cmPhe^3) were added to this model. The turn comprising atoms, -CO(cmPhe), Glu, Asn, NH(Val)-, and the protein atoms were kept fixed during minimization and simulated annealing (SA). 75 SA cycles were run with a different random seed for each cycle. In each SA cycle the same minimized starting geometry was subjected to an MD simulation at 2000K for 10 ps and then cooled in 5K decrements to 5K during 195 ps. The final structure was minimized and stored.

The structure with the lowest energy out of the 75 obtained structures served as the starting geometry for three 100 ps MD (NVT-ensemble) simulations at 298 K. Three different random seed numbers were used, and the complex was solvated by a sphere of water molecules with the radius of 22 Å, centered around the Cα of Glu^4 of G1TE(Adi^1, cmPhe^3). During minimizations and simulations, all atoms were held fixed except the ligand, the water molecules within a radius of 18 Å around the Cα of Glu^4 of G1TE(Adi^1, cmPhe^3), and the sidechains of Grb2-SH2 within 6 Å around G1TE(Adi^1, cmPhe^3). The coordinates were saved every 1 ps and minimized by 300 steps of CG-PR. The frame with the lowest energy of the 300 obtained solvated structures was identified and is depicted in Fig. 2.

Inhibition of Grb2 interaction with Tyr-phosphorylated p185(erbB-2). Cell lysates were prepared from serum-treated erbB2 overexpressing breast cancer cells (MDA-MB-453), as described previously (14). Cell lysates were treated with G1TE(Adi^1, cmPhe^3) or control peptide G1TE(Ala^3) at various concentrations for 30 min, and then 500 µg of protein was immunoprecipitated with anti-Grb2 antibody (Santa Cruz Biotech., Santa Cruz, CA, USA) and collected with protein A Sepharose. Immunoprecipitated proteins were separated by SDS-PAGE on 8-16% gradient gels (Novex, San Diego, CA, USA). pTyr-containing proteins were detected by Western blotting using anti-phosphotyrosine antibodies (Upstate Biotechnology, Lake Placid, NY, USA) and visualized with ECL (Amersham, Arlington Heights, IL). Previous experiments have shown that a major tyrosine phosphorylated protein in these cells is the p185$^{erbB\text{-}2}$, which is overexpressed as a consequence of gene amplification (22). To evaluate the equal loading of the proteins, blots were subsequently re-probed with a monoclonal antibody recognizing the total Grb2 protein. A semi-quantitation of the Grb2 associated p185 $^{erbB\text{-}2}$ band was done with densitometer and expressed as percent of control in the untreated cells.

Table 2. Grb2-SH2 Domain Inhibitory Activity of the Peptides **1-14**^{a}. Variation in X^3 and X^1.

Compound	Substitute of Tyr^3	Analog term	IC_{50} (µM)
1	Tyr	G1TE	20 ± 5
2	Deleted	G1TE (no Tyr^3)	> 1000
3	Ala	G1TE (Ala^3)	> 1000
4	Htyr	G1TE (hTyr^3)	At 1000 µM, 40% inhibition
5	Phe	G1TE (Phe^3)	94.5 ± 10.5

6	PfPhe	G1TE ($pfPhe^3$)	> 1000
7	Adi	G1TE (Adi^3)	600
8	pTyr	G1TE ($pTyr^3$)	0.13 ± 0.01^b
9	F_2Pmp	G1TE (F_2Pmp^3)	0.88 ± 0.09
10	F_2cmPhe	G1TE (F_2cmPhe^3)	5.7 ± 0.5
11	malTyr	G1TE ($malTyr^3$)	6.0 ± 0.4
12	cmPhe	G1TE ($cmPhe^3$)	1.15 ± 0.05
13	CmPhe (Ala^1 mutant)	G1TE (Ala^1, $cmPhe^3$)	1.65 ± 0.15
14	CmPhe (Adi^1 mutant)	G1TE (Adi^1, $cmPhe^3$)	0.70 ± 0.12

[a]The experiments were performed on a BIAcore 2000 instrument by the method described previously (14). The results represent mean value of at least two independent experiments and are expressed as the concentration at which half-maximal inhibition (IC_{50}) of binding of Grb2-SH2 to biotinylated DDPSpYVNVQ was observed. IC_{50} of reference SHC(pY317) peptide: 1.0±0.2 μM.
[b]This value has previously been reported (23), and is used here as a reference.

RESULTS AND DISCUSSION:

Tyrosine 3 in G1TE is required for Grb2-SH2 binding. - The phage library derived nonphosphorylated cyclic peptides, typified by G1TE, **1**, comprise a unique family of agents binding to the SH2 domain of the intracellular adapter protein Grb2 (14,16). Earlier Ala mutation studies demonstrated that essentially all amino acids are required for good binding affinity, especially Tyr and Asn in the consensus sequence Y^3-X-N^5 in G1TE. Our recent structure / activity studies amply demonstrated that the carboxyl sidechain of Glu in position 1 of G1TE partially compensates for the absence of the phosphate group on Tyr^3 (23). In order to assess the importance of the consensus tyrosine in these peptides and then help design potent non-phosphorylated inhibitors, we synthesized a series of G1TE analogs substituted at the Tyr^3 position and evaluated their binding affinity to the Grb2-SH2 domain with the Biacore binding assays (Table 2). As shown in the table, deletion (**2**) or Ala substitution (**3**) of Tyr^3 eliminates the inhibitory activity, which strongly suggests that Tyr^3 is a very important determinant for high affinity binding of the non-phosphorylated peptide for the Grb2 SH2 domain. But just extending the side-chain of Tyr with one CH_2 moiety, i.e. substitution of Tyr with homotyrosine (hTyr) substantially diminishes the binding (**4**), which indicates the binding affinity is very sensitive to the positioning of phenyl ring or the phenolic hydroxyl group of the sidechain. Comparison of the potency loss on Phe (**5**, IC_{50} = 94.5±10.5 μM) and the non-aromatic α-amino-adipate (Adi) replacements (**7**, IC_{50} = 600 μM) for Tyr^3 further confirms that important sidechain interactions can be achieved only when the functional sidechain is positioned precisely to fit the binding pocket of the Grb2-SH2 domain. The Phe^3 mutant still sustains moderate binding to SH2, and this points out the functional importance of the aromatic sidechain itself at position 3, regardless of the loss of polar substituents. In comparison, the pentafluoro-Phe replacement (**6**) abolishes the binding. Both the increase in hydrophobicity and the reduced electron density of the phenyl ring as a result of the strong electron-withdrawing fluorine substituents appear to disfavor association within the protein binding pocket (24). Remarkably, our molecular modeling studies

of nonfluorinated G1TE peptide / Grb2-SH2 complex, as well as the X-ray structure of the phosphopeptide / Grb2-SH2 complex (20) indicate that the polar sidechains of Arg67 and Lys109 of the protein are in a position to form aromatic-π / cation interactions with pTyr or Tyr of the peptides. It is known that fluorination of the aromatic ring disrupts such interactions (24). These results farther corroborate the importance of cation / π interactions in the Tyr or pTyr binding pocket of Grb2-SH2 domain and presumably in the related Src-SH2 protein also (25). We have carried out *ab initio* calculations (Gaussian 94, HF/6-31G**, in vacuum) to estimate the difference in interaction energy in a simple model system. For benzene/NH_4^+ a favorable geometry was found with the ammonium ion located 3 Å above the center of the plane of benzene ring, with a favorable interaction energy of –15 kcal. In contrast, the analogous single point energy calculation of a complex of geometry-optimized pentafluorobenzene and NH_4^+ showed that this complex was disfavored by 4 kcal. From the results above, we can conclude that optimally positioned phenyl moiety and to some extent the polar phenolic 4-hydroxyl functionality are required for effective binding of this non-phosporylated inhibitor.

[Figure 2] Figure 2. A. Complex of the cyclic thioether peptide G1TE(Adi^1, cmPhe^3), **14**, with the Grb2-SH2 domain protein, based on molecular modeling. *Note*: the solid green stick structure corresponds to the peptide ligand. **B.** Schematic representation of the ionic and/or polar interactions between the –Glu^1-Leu-cmPhe^3-Glu-Asn^5- segment of the peptide ligand **14,** based on molecular modeling. **C.** Schematic representation of ionic and/or polar interactions in a complex of the Bcr-Abl phosphopeptide and the Grb2-SH2 domain, based on X-ray sructural information (20). *Note:* not all interactions are indicated.

Tyrosine phosphate mimics in G1TE provide efficient inhibitors of Grb2-SH2 domain interactions. - Predictably, phosphorylation of Tyr^3 in the cyclic peptide, **1,** greatly improved the Grb2-SH2 binding affinity, by a factor of 150 fold (**8**, IC_{50} = 0.13±0.01 μM). However, phosphotyrosine itself is not a desirable building block for *in vivo* active inhibitor design, due to the hydrolytic lability of the phosphate ester toward phosphatases and the poor membrane penetration of the doubly ionized phosphate group.

Our subsequent efforts focus on examining the effect and potential use of phosphotyrosyl mimetics as replacements of Y^3 on the binding efficacy to Grb2-SH2 domain in the context of this novel non-phopshorylated peptide ligand.

F_2Pmp was reported earlier to be a good phosphatase-resistant surrogate for the pTyr residue (26). Incorporation of F_2Pmp into position 3 of **1** results in submicromolar affinity inhibitor (**9**, IC_{50} = 0.88±0.09 μM), but reduces the potency by 6-fold relative to G1TE(pTyr^3), which was contrary to the finding of F_2Pmp retaining as good binding affinity as pTyr in pTyr-containing short peptide inhibitors of various SH2 domains (26). The reduction in potency of F_2Pmp might be attributed to the loss of interactions with the pTyr ester oxygen which was replaced by a difluoromethylene unit in F_2Pmp, and in our case, the fluorines added in the bridging methylene can not restore the lost interactions, as was previously observed in phosphopeptides (26). Even with the retention of the bridging oxygen, O-malonyl-L-tyrosine replacement for Tyr^3 in G1TE does not remarkably increase the inhibitory activity (**12,** IC_{50} = 6.0±0.4 μM). This might result

from non-optimal positioning of the carboxyl groups because of a too long linking side-chain. These results further confirm the notion that the nonphosphorylated cyclic peptide ligand **1** requires more specific conformation and interactions with the binding pocket of the protein, most likely involving the side chains of nearby residues e.g. E^1, Y^3, E^4, N^5 and M^8.

4-Carboxymethyl-L-phenylalanine (cmPhe) and 4-carboxydifluoromethyl-L-phenylalanine (F_2cmPhe) were successfully utilized previously as non phosphorus-containing pTyr mimetics in a high affinity β-bend mimicking platform (27, 28), and in this case, substitutions of cmPhe and F_2cmPhe for Tyr^3 in **1** resulted in the binding potency enhancement by 17-fold (**12**, IC_{50} = 1.15±0.05 μM) and 4-fold (**10**, IC_{50} b= 5.7±0.5 μM), respectively, relative to the parent peptide. The introduction of fluorine into the cmPhe residue reduced the SH2 domain affinity as was also observed in the β-bend mimicking structures (27). A further development was based on our recent discovery of the significant overlapping roles of Glu^1 and the $pTyr^3$ side chains in **1** (23). We found that extending the Glu^1 sidechain with an additional methylene group by using α-amino-adipate (Adi) improved the binding affinity by 6-fold in G1TE (23). In accordance with this, by substituting Adi for Glu^1 in **12**, we obtained submicromolar non-phosphorylated cyclic peptide inhibitor of Grb2-SH2 domain (**14**, IC_{50} = 0.70±0.12 μM), whereas Ala^1 mutant **12** showed only a slightly reduced binding potency (**13**, IC_{50} = 1.65±0.15 μM).

The molecular model for peptide **14** bound to Grb2-SH2 (Fig. 2) was generated based on the assumption of a β-turn for the inhibitory peptide segment, pTyr (or its mimic)-Glu-Asn-Val, as was described in the Methods Section. In the pTyr pocket the orientation and the hydrogen bonding pattern of cmPhe is consistent with previous modeling results for cmPhe containing peptides bound to Grb2-SH2 (27), and it is also in good agreement with the orientation and the hydrogen bonding pattern of cmPhe in the crystal structure of Ac-cmPhe-Glu-Glu-Ile bound to lck-SH2 domain (28). In that particular X-ray structure of lck-SH2 domain the BC loop has a different conformation, and one of the sidechain carbonyl oxygens interacts with the backbone nitrogen of Glu157, instead of the Ser156 sidechain. The latter Ser is equivalent to Ser88 in Grb2-SH2, which participates in interactions with the ligand in our case (Fig. 3a, and 3b). Adi^1 of G1TE(Adi^1, $cmPhe^3$) interacts with Ser90 and Lys109 of the protein, but its sidechain is located only in the periphery of the pTyr pocket, probably due to the repulsion between the side chains of Adi^1 and $cmPhe^3$. The lesser importance of Adi in this pTyr-mimic containing peptide is reflected by the very moderate loss of binding affinity in the G1TE (Ala^1, $cmPhe^3$) mutant, **13**.
G1TE(Adi¹, cmPhe³) blocks Grb2-SH2 domain function in cell homogenates. The Grb2 acts as an intracellular adaptor protein in transmitting activated growth factor receptor signaling by forming association with the nucleotide exchange factor, the Sos protein. In the breast cancer cell line, MDA-MB-453, the oncogenic $p185^{erbB\text{-}2}$ receptor protein is constitutively overexpressed and phosphorylated (22). In order to evaluate the inhibitory effectiveness of our agents under near-physiological conditions, we carried out assays in cell homogenates. Peptide **14,** G1TE(Adi^1, $cmPhe^3$) was selected for this purpose, which showed sub-micromolar binding affinity to the Grb2 target protein in our *in vitro* SPR based competitive binding assays (Table 2). Various concentrations of the peptide were incubated with cell lysates of the breast cancer cell line MDA-MB-453, and the Grb2/$p185^{erbB\text{-}2}$ complexes were immunoprecipitated with anti-

Grb2 antibody. Western blot analysis demonstrated that peptide **14** effectively inhibited this protein association in the 0.4 to 2 μM concentration range in a dose dependent manner (Figure 3). In comparison, the Ala^3 mutant control peptide **3** was ineffective at two orders of magnitude higher concentration. These results, and our earlier report (14), demonstrate that cyclic peptides that are effective in *in vitro* SPR assays are also effective in inhibiting a key protein association process involving the oncogenic constitutively expressed erbB-2 receptor association with the Grb2 adapter protein that then mediates a key cellular activation process.

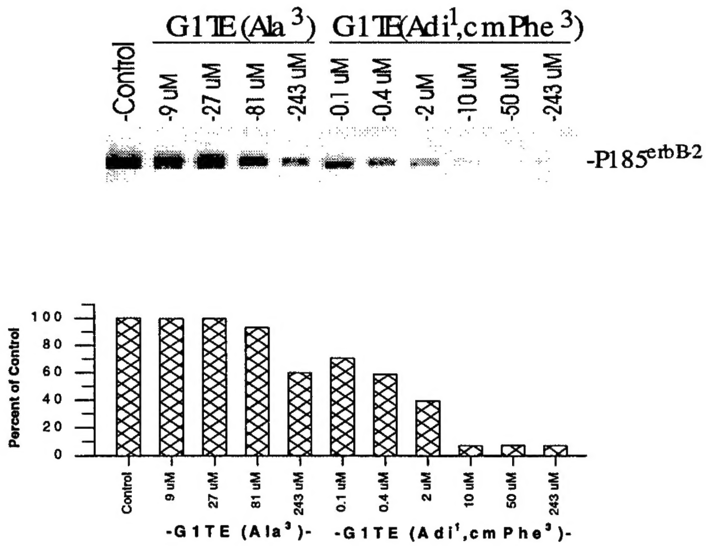

Figure 3. Inhibition of complexes formed between the Grb2 protein and GF-receptor protein $p185^{erbB\text{-}2}$ in MDA-MB-453 cell homogenates, on treatment with peptide **3**, G1TE(Ala^3) as control at a concentration range of 9 – 243 μM, and peptide **14**, G1TE(Ala^1, $cmPhe^3$) at a concentration range of 0.1 – 243 μM.

KEY RESEARCH ACCOMPLISHMENTS: Bulleted list of **key** research accomplishments emanating from this research.

- Design and synthesis of cyclic and other modified Geb2 peptide inhibitors with improved activities over G1TE;
- Biochemical and biological studies of the modified Grb2 peptide inhibitors;
- Provided the proof-of-concept for the small molecule inhibitor studies of Grb2 interruption and also the success of funding from Komen Foundation for Breast Cancer.
- Provided more potent peptides for further cell based inhibition studies.

REPORTABLE OUTCOMES: Provide a list of reportable outcomes to include:

– manuscripts, abstracts, presentations;

1. Ya-Qiu Long, Zhu-Jun Yao, Feng-Di T. Lung, Johannes H. Voigt, C. Richter King, Terrence R. Burke, Jr., Juliet H. Luo, **Dajun Yang**, and Peter P. Roller, Structural Requirements for Tyr in the

Consensus Sequence Y-E-N of a Novel Non-phosphorylated Inhibitor to the Grb2-SH2 Domain, **Biochem. Biophys. Res. Commun.**, 264:902-908, 1999.

2. Long, Y.Q., Lung, F.D., Voigt, J., Yao, Z., Burke, T.R., **Yang, D.**, Luo, J., Guo, R., King, C.R., and Roller, P.P. High Affinity nonphosphorylated cyclic peptide inhibitors of Grb2-SH2/growth factor receptor interaction. "Peptides for the New Millennium: proceedings of the 16th American Peptide Symposium", G. B. Fields, J.P. Tam, and G. Barany (Eds.), Kluwer Academic Publishers, Dordrecht, The Netherlands, 102-105, 1999.
3. *Terrence R. Burke, Jr., Juliet Luo, Zhu-Jun Yao, Yang Gao, He Zhao, George W.A. Milne, Johannes H. Voigt, C Richter King and **Dajun Yang***. Monocarboxylic-based phosphotyrosyl mimetics in the design of Brb2 SH2 domain inhibitors. **Bio. Med. Chem. Lett.**, 9, 347-352, 1999.
4. *Burke, T.R, Gao, Y., Yao, Z., Voigt, J., Luo, J., and **Yang, D.** Potent Non Phosphate-Containing Grb2 SH2 Domain Inhibitors. Peptide Science, 20: 49-52, 1999.
5. *Gao, Y., Yao, Z., Voigt, J., Luo, J., **Yang, D.** and Burke, T.R. Novel phosphotyrosyl mimetics for the preparation of potent small molecule Grb2 SH2 domain inhibitors. "Peptides for the New Millennium: proceedings of the 16th American Peptide Symposium", G. B. Fields, J.P. Tam, and G. Barany (Eds.), Kluwer Academic Publishers, Dordrecht, The Netherlands, 10-13, 1999.

*Those are related publications in the same target use small molecule approaches.

- patents and licenses applied for and/or issued;

  One patent application has been filed jointly with NIH on the modified peptide inhibitors.

- degrees obtained that are supported by this award;

  Not applicable.

- development of cell lines, tissue or serum repositories;

  Not applicable.

- informatics such as databases and animal models, etc;

  Not applicable.

- funding applied for based on work supported by this award;

  Susan G. Komen Breast Cancer Foundation

  Principal Investigator

  Title: Grb2 Targeted Therapeutics for Breast Cancer

  1999-2001, annual direct $100,000.00, annual indirect $25,000

- employment or research opportunities applied for and/or received on experiences/training supported by this award.

  Not Applicable.

CONCLUSIONS: Summarize the results to include the importance and/or implications of the completed research and when necessary, recommend changes on future work to better address the problem. A "so what section" which evaluates the knowledge as a scientific or medical product shall also be included in the conclusion of the annual and final reports.

By incorporating a series of various structurally relevant amino acids in position Y^3 of the cyclic peptide G1TE, we explored the functional importance and structural requirement for Tyr^3. Phenyl moiety and polar group with specific orientation in position Y^3 are particularly required for high affinity binding of G1TE to its cognate protein, the Grb2-SH2 domain. Substitutions of

pTyr mimetics for Tyr^3 provide low micromolar to submicromolar affinity inhibitors. The combination of cmPhe replacement in position Y^3 and Adi substitution in position E^1 results in a potent non-phosphorylated cyclic peptide antagonist of Grb2-SH2 domain with $IC_{50} = 0.70 \pm 0.12$ μM. When pTyr containing peptides bind to various SH2 domains of proteins, the pTyr binding pocket provides the driving force for binding affinity. The Grb2-SH2 domain is unique, in that ligands binding to it are required to possess turn conformations, such that pTyr, and Asn or its mimic in the pTyr + 2 position, are required to fit into specific receptor sites (29). The merit of the G1TE family of nonphosphorylated cyclic peptides is that for high affinity binding they require additional well defined conformational space, in order to accommodate the requisite interactions with the acidic sidechains of Glu or Gla in the Tyr – 2 position of the peptide, as our previous work demonstrated (23). These results provide a better understanding of the molecular binding mode of this novel non-phosphorylated cyclic peptide ligand, and suggest new strategies for designing potent and specific non-phosphorylated inhibitors of Grb2-SH2 domain. Very recently the discovery of a number of non-phosphorylated cyclic peptides was disclosed, using phage library methodology, that showed low micromolar binding affinities to Grb2-SH2 (30). These structural variants have in common with our peptides the strong selection of Glu at the Tyr – 2 position. These additionl variants will provide additional possibilities for rational design of adventageous peptides and peptidomometics.

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APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples of appendices include journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

We will mail these appendices separately.

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